IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Docket No.: FUERTES-LOPEZ

In re PATENT Application of:	)	
LAURA FUERTES-LOPEZ & MARCOS TIMÓN-JIMENEZ	)	**Examiner: Anne Maria Sabrina Wehbe**
Appl. No.: 10/816,591	)	**Group Art Unit: 1633**
Filed: April 1, 2004	)	**Confirmation No.: 8510**
For: DNA EXPRESSION CONSTRUCT FOR THE TREATMENT OF INFECTIONS WITH LEISHMANIASIS	)	

DECLARATION OF MARCOS TIMON-JIMENEZ

CONSIDERED: /AW/ (03/30/2009)

Marcos Timon-Jimenez hereby deposes and states:

1 I am one of the inventors in the above identified patent application and I make this declaration to support the proposition that experiments that lead to our invention, the DNA expression construct for the treatment of infections with Leishmaniasis showed unexpected results.

2. Currently I am an employee of the Spanish Medicines Agency. My CV showing my background which is incorporated herein by references is attached hereto as Exhibit 1.

3. The experiments which are the basis for the patent application were made when I was a Chief Scientific Officer at Mologen Molecular Medicines located in Madrid, Spain. A scientific paper published in *Vaccine 21 (2002) 247-257* and

showing our team's construction of the MIDGE vector and its efficacy in an immune response is attached hereto as Exhibit 2. In the following paragraphs I refer to the results as shown in the Figures and Tables of the paper.

4. It is known that Leishmaniasis constitutes a major health problem. The possibility of developing a vaccine was postulated by us based on our awareness that patients that have overcome the infection develop strong immunity.

5. We investigated whether modification of the MIDGE vector with covalently linked peptides was able to favorably change the immune response. We conducted a vaccination trial in mice using different gene expression constructs all of which encoded the p36 LACK: MIDGEp36-NLS, plasmid (pMOKp36) and recombinant vaccinia virus (rVVp36). All were applied in different vaccination regimes followed by experimental challenge with *Leishmania major* promastigotes, as seen in Table 2. Our parameter for the clinical success of the vaccination was measurement of the growth of the infection related lesions in the infected hind paw of the mice. As another parameter the IgG1 and IgG2a antibody subtypes were determined since we knew that antibody titres alone cannot be taken as an indication of a supposed protective effect, but the ratio of these two isotypes is an indicator of whether a protective or a non-protective immune response against this particular disease has been elicited.

6. We surprisingly found that two immunizations with MIDGEp36-NLS induced a shift from a Th2 towards a Th1 response comparable to that induced by the protocol consisting on a first immunization with the plasmid pMOKp36 followed by a second immunization with rVVp36. To assess the conferred

protection we therefore conducted a challenge infection in the footpad of mice using *Leishmania major* promastigotes which in the mouse strain used (Balb/c) is a well described model of cutaneous leishmaniasis. Success was rated in accordance with the growth progression of the lesions. The results are seen in Fig. 2 (A) and (B) showing that mice immunized with the MIDGEp36-NLS/MIDGEp36-NLS showed the smallest lesions indicating that this vaccination regime confers the best protection against the experimental infection with this parasite.

7. The unexpected results of these experiments showed that immunization of mice with MIDGE vectors coding for P36/LACK induce the same level of protection against challenge with *L. major* than equimolar amounts of plasmid coding for the same antigen. Most significantly, two doses of NLS-modified MIDGE conferred full protection against the same antigen, resembling the results obtained by priming with plasmid DNA and boosting with rVV, both expressing LACK as seen in Fig. 2. This was an unexpected result in that the pharmaceutical composition used was shown to be superior in producing a protective effect than the best vaccination protocols available. As shown in Exhibit 2, that vaccination protocol is based on a primary immunization (priming) with a recombinant plasmid followed by a secondary immunization (boosting) with a recombinant vaccinia virus both coding for the same antigen (in this case *Leishmania* p36/LACK), known at that time and deemed the state of the art. Thus, our pharmaceutical composition, consisting on a MIDGE vector coding for the LACK antigen and modified with the NLS peptide and used in a similar

vaccination regime (priming and boosting), showed to have a better protective effect and avoids the potential side effects of plasmids and recombinant virus shuttles, aside from the simplicity of production and application.

I declare under penalty of perjury under the laws of the United States of America that the foregoing is true and correct.

Date: 08/03/08

Dr. Marcos Timon-Jimenez

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RELEVANT PROFESIONAL EXPERIENCE

Position: **Quality Assessor**
Date: August 2005–Present
Organization: Department of Biological Products and Biotechnology, Spanish Medicines Agency
Location: Madrid, Spain

Position: **Scientific Director**
Date: August 2000–July 2005
Organization: Mologen Molecular Medicines S. L.
Location: Madrid, Spain

Position: **Research Scientist**
Date: October 1996-August 2000
Organization: The Edward Jenner Institute for Vaccine Research
Location: Compton (near Oxford), Berkshire, UK

Position: **Research Fellow**
Date: October 1995-September 1996
Organization: Department of Oncology, University College
Location: London, UK

Position: **Post-doctoral Scientist**
Date: September 1993-September 1995
Organization: Tumour Immunology Unit, Imperial Cancer Research Fund (now Cancer Research UK)
Location: London, UK

Position: **PhD Student**
Date: 1988-1993
Organization: Department of Immunology, Hospital 12 de Octubre
Location: Madrid, Spain

QUALIFICATIONS

- **2007: Specialist in Immunology**
- **1993: PhD**

Universidad Complutense, Madrid, Spain

- **1982-1987: BsC in Biology (Biochemistry and Genetics)**

Universidad Complutense, Madrid, Spain

LANGUAGES

- **Spanish**

Mother tongue

- **English**

Perfectly fluent

- **Others**

French and German at basic level

RESPONSABILITIES AT PRESENT POST

- To **assess** dossiers for the qualification of **Investigational Medicinal Products**.

- To **assess** dossiers for the **marketing authorization** of biotechnological products for use as human medicines by the **centralized procedure (EMEA)**.

- To **participate** in the **Biotechnology Working Party** and the **Gene Therapy Working Party** at the **EMEA**.

RESPONSABILITIES AT PREVIOUS POST (MOLOGEN)

- To **direct, plan** and **supervise** all **basic research** related to the use of the company's two major platform technologies (the MIDGE DNA vectors and the dSLIM immunomodulator).

- To find and develop **applications** for both technologies, mainly on the following fields: **human vaccines, veterinary vaccines, cancer immunotherapy**.

- To direct the **development** of a **vaccine** against **human** and **canine Leishmaniosis** based on the MIDGE technology.

- To **support** and **advice** the **Business Development** team on selling the technologies and their applications.

- The **group** under my direct **supervision** included: two post-doctoral scientists, two PhD students, one graduate student and six research assistants, plus several practical students every year.

PRACTICAL EXPERIENCE

SCIENTIFIC EXPERIENCE: 17 years experience in the field of **Immunology**, from basic to applied research. I have worked in public institutions, charities, public-private centres and industry. Apart from Immunology, I have a strong theoretical and practical knowledge of many other fields, including:

- Molecular Biology
- Cell Biology
- Microbiology
- Virology
- Animal experimentation
- Vaccine development

BUSINESS DEVELOPMENT: At Mologen I always worked very closely with the Business Development team. I presented the technologies and the products based on them at most of the major pharmaceutical companies, many scientific meetings and research institutes around the world, including NIH (Bethesda), IDRI (Seattle), Max Planck Institute (Berlin), etc.

REGULATORY ISSUES: Good knowledge of **regulatory issues** established by **EMEA**. For the last two years at Mologen, one of my main activities was the **development** of a **vaccine** against Leishmaniosis based on MIDGE vectors. To plan the development of this vaccine, we visited the regulatory authorities and the World Health Organization on several occasions. At my **current post**, the main activity is to **evaluate biotechnological products** for their suitability as medicines for humans as well as the active contribution to the Biologics and the Gene Therapy Working.Parties of the EMEA.

PATENTS

1-Timón M, López-Fuertes L. DNA-expression construct for treatment of infections with leishmaniasis.

2-Timón M, Moreno S. Means for improving immune response.

3-Wittig B, López-Fuertes L, Timón M. Mittel zur behandlung von infektionen mit Leishmaniose.

THESIS SUPERVISION

- **Student:** Sonia Moreno López
- **Title:** Characterisation of the immune response induced by Minimalistic, Immunogenically Defined Gene Expression (MIDGE) vectors as vehicles for DNA vaccination
- **Place where work was carried out:** Mologen Molecular Medicines. Madrid, Spain
- **Date and place where thesis was presented:** July 2004, Universidad Autónoma, Madrid, Spain. **European Doctorate.**

- **Student:** Esther Díaz del Pozo
- **Title:** Transcriptional control of the human leukocyte common antigen using the Japanese pufferfish *Fugu rubripes* as a model genome
- **Place where work was carried out:** The Edward Jenner Institute for Vaccine Research, Compton, UK
- **Date and place where thesis was presented:** January 2001, University College, London, UK. **Co-directed with Prof. Peter Beverley.**

SCIENTIFIC PUBLICATIONS

1-Moreno S, Sack F, Wittig B, de Andrés X, Schmidt M, Schroff M, Timón M. Modulation and potentiation of the immune response to DNA vaccines by MIDGE vectors coupled to peptides. 2007. Submitted.

2- Rodríguez-Cortés A, Ojeda A, López-Fuertes L, Timón M, Altet L, Solano-Gallego L, Sánchez-Robert E, Francino O, Alberola J. Vaccination with plasmid DNA encoding Kmp-11, TSA, LACK and gp63 does not protect dogs against *Leishmania infantum* experimental challenge. *Vaccine* 2007; 25:7962-7971.

3-Rodríguez-Cortés A, Ojeda A, López-Fuertes L, Timón M, Altet L, Solano-Gallego L, Sánchez-Robert E, Francino O, Alberola J. A long term experimental study of canine visceral leishmaniasis. *Int. J. Parasitol.* 2007; 37:683-693.

4-Hoffmann F, Timón M, Wittig B. Gene transfer in corneal transplantation. *In* Progress in gene therapy research. Redberry GW ed. Nova Science Publishers, Inc. New York. 2005.

5-Moreno S, López-Fuertes L, Vila-Coro AJ, Konig, SA, Wittig B, Schroff M, Juhls C, Junghans C, Timón M. DNA immunisation with minimalistic expression constructs. *Vaccine* 2004; 22:1709-1716.

6-Moreno S, Timón M. DNA vaccination: an immunological perspective. *Inmunología* 2004; 23:41-55.

7-López-Fuertes L, Pérez-Jiménez E, Vila-Coro AJ, Sack F, Moreno S, Konig SA, Junghans C, Wittig B, Timón M, Esteban M. DNA vaccination with linear minimalistic (MIDGE) vectors confers protection against *Leishmania major* infection in mice. *Vaccine* 2002; 21 (3-4): 247-257.

8-Timón M, Beverley PCL. Structural and functional analysis of the human CD45 gene (PTPRc) upstream region: evidence for a functional promoter within the first intron of the gene. *Immunology* 2001; 102:180-189.

9-Díaz del Pozo E, Beverley PCL, Timón M. Genomic structure and sequence of the leukocyte common antigen (CD45) from the pufferfish *Fugu rubripes* and comparison with its mammalian homologue. *Immunogenetics* 2000; 51:838-846.

10-Goff LK, van Soest S, Timón M, Tchilian E, Beverley PCL. Protein tyrosine phosphatase receptor type C polypeptide (PTPRC) on human chromosome band 1q31.q32 localizes with marker D1S413 on a 610-Kb yeast artificial chromosome. *Cytogenet. Cell Genet.*1999; 87:223-224.

11-Zilch CF, Walker AM, Timón M, Goff LK, Wallace DL, Beverley PCL. A point mutation within CD45 exon A is the cause of variant CD45RA splicing in humans. *Eur. J. Immunol.* 1998; 28: 22-29.

12-Timón M, Elgar G, Habu S, Okumura K, Beverley PCL. Molecular cloning of major histocompatibility complex class I cDNAs from the pufferfish *Fugu rubripes*. *Immunogenetics* 1998; 47:170-173.

13-Timón M, Beverley PCL. Identification of an intragenic promoter in the human CD45 gene. *Biochem. Soc. Trans.* 1997; 25: S177.

14-Rodríguez-Gallego C, Corell A, Pacheco A, Timón M, Regueiro JR, Allende LM, Madroño A, Arnaiz-Villena A. Herpes-virus saimiri transformation of T cells in CD3γ immunodeficiency: phenotypic and functional characterization. *J. Immunol. Methods* 1996; 198: 177-186.

15-Martin-Villa JM, Luque I, Martínez-Quiles N, Corell A, Regueiro JR, Timón M, Arnaiz-Villena A. Diploid expression of human leukocyte antigen class I and class II molecules on spermatozoa and their cyclic inverse correlation with inhibin concentration. *Biol. Reprod.* 1996; 55:620-629.

16-Arnaiz-Villena A, Rodríguez-Gallego C, Timón M, Corell A, Pacheco A, Alvarez-Zapata D, Madroño A, Iglesias P, Regueiro JR. Diseases involving the T-cell receptor/CD3 complex. *Crit. Rev. Oncol/Hematol.* 1995; 19:131-147.

17-Rodríguez-Gallego C, Arnaiz-Villena A, Corell A, Manazanares J, Timón M, Pacheco A, Regueiro JR. Primary T lymphocyte immunodeficiency associated with a selective impairment of CD2, CD3, CD43 (but not CD28)-mediated signal transduction. *Clin. Exp. Immunol.* 1994; 97: 386-391.

18-Góngora R, Corell A, Regueiro JR, Carasol M, Rodríguez-Gallego C, Paz-Artal E, Timón M, Allende L, Arnaiz-Villena A. Pripheral blood reduction of memory (CD29+,CD45R0+, and "bright" CD2+ and LFA-1+) T lymphocytes in Papillon-Lefevre Syndrome. *Human Immunol.* 1994; 41: 185-192.

19-Timón M, Arnaiz-Villena A, Ruiz-Contreras J, Ramos-Amador JT, Pacheco A, Regueiro JR. Selective impairment of T lymphocyte activation through the T cell receptor/CD3 complex after Cytomegalovirus infection. *Clin. Exp. Immunol.* 1993; 94:38-42.

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22-Alarcón B, Terhorst C, Timón M, Arnaiz-Villena A, Pérez-Aciego P, Regueiro JR. Congenital T-cell receptor immunodeficiencies in man. *In* Immunodeficiencies. Rosen FS, Seligmann eds. Harwood Academic, Chur (Switzerland), pp 155-166, 1993.

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24-Arnaiz-Villena A, Timón M, Rodríguez-Gallego C, Pérez-Blas M, Corell A, Martín-Villa JM, Regueiro JR. Human T-cell activation deficiencies. *Immunol. Today* 1992; 13:259-265.

25-Arnaiz-Villena A, Timón M, Corell A, Pérez-Aciego P, Martín-Villa JM, Regueiro JR. Primary immunodeficiency caused by mutations in the gene encoding the CD3γ subunit of the T-lymphocyte receptor. *N. Engl. J. Med.* 1992; 327:529-533.

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27-Regueiro JR, Pérez-Aciego P, Timón M, Morales P, Pérez-Blas M, Arnaiz Villena A. T cell function in patients with impaired antibody responses to polysaccharide antigens. Reply. *Eur. J. Immunol.* 1991; 21: 2293-2296.

28-Timón M, Arnaiz-Villena A, Pérez-Aciego P, Morales P, Benmamar D, Regueiro JR. A diallelic RFLP of the CD3-epsilon chain of the clonotypic T-lymphocyte receptor is not associated with certain autoimmune diseases. *Hum. Genet.* 1991; 86:363-364.

SCIENTIFIC MEETINGS

Many poster and oral presentations. Some of the most recent and relevant oral presentations as **invited speaker** are listed below:

1- New approaches to vaccine development: from the bench to the field. Berlin, Germany, 8-10 September 2005.

2-Third world congress on Leishmaniosis. Palermo, Italy, 10-15 April 2005.

3-DNA vaccines 2004 conference. Monte Carlo, Monaco, 17-19 November 2004

4-Modern vaccines adjuvants and delivery systems. Dublin, Ireland, 4-6 June 2003.

5-Gene-based vaccines: mechanisms, delivery systems and efficacy. Breckenridge, Colorado, USA, 10-15 April 2002.

ELSEVIER

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DNA vaccination with linear minimalistic (MIDGE) vectors confers protection against *Leishmania major* infection in mice

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Abstract

Immunization protocols based on priming with plasmid DNA and boosting with recombinants of vaccinia virus (rVV) encoding the same antigen offer great promise for the prevention and treatment of many parasitic and viral infections for which conventional vaccination has little or no effect. To overcome some of the potential problems associated to the use of plasmids, we have developed minimalistic, immunogenically defined, gene expression (MIDGE®) vectors. These linear vectors contain only the minimum sequence required for gene expression and can be chemically modified to increase the immune response. Here, we demonstrate that MIDGE vectors coding for the LACK antigen confer a highly effective protection against *Leishmania* infection in susceptible Balb/c mice. Protection is achieved at lower doses of vector compared to conventional plasmids. This efficacy could be greatly improved by the addition of a nuclear localization signal (NLS) peptide to the end of the MIDGE vector. In fact, immunization with two doses of NLS-modified MIDGE conferred similar or even better protection than that achieved by priming with plasmid DNA followed by boosting with rVV. These results demonstrate that MIDGE vectors are a good alternative to plasmid and rVV for immunization.

Keywords: MIDGE; DNA vaccine; *Leishmania*

1. Introduction

Leishmaniasis is a disease with different clinical manifestations produced by Trypanosomatidae of the genus *Leishmania*. Depending on the host immune response, the strain and the virulence of the parasite, the clinical manifestations extend from self-healing cutaneous lesions to the visceral form of the disease, the latter being fatal if untreated. Worldwide there are 2 million new *Leishmania* cases each year and the disease is endemic through parts of Africa, southern Europe and Central and South America [1]. With the advent of the HIV epidemic, leishmaniasis has surged as a reactivated infection in AIDS patients in many countries. Moreover, in Spain and the southwest of Europe leishmaniasis is zoonotic and dogs are the main reservoir host. Epidemiology of the disease revealed that between 10 and 37% of dogs in the Mediterranean area are infected and develop the visceral form of the disease [2].

Eradication of the disease has proven difficult. Chemotherapy has only a modest effect and there is no effective and safe vaccine against any form of clinical leishmaniasis. However, individuals who recovered naturally from infection develop strong immunity against re-infection suggesting that vaccination against leishmaniasis is feasible. Infection of inbred strains of mice with *Leishmania major* provides the best model for the immunoregulation that occurs during a cell mediated response to this intracellular pathogen [3]. Using the *Leishmania major* model, studies demonstrated that the generation of protective immunity against *L. major* is T cell and cytokine mediated [4]. Expansion of a Th1 subset of $CD4^+$ T lymphocytes secreting IFN-γ and IL-12 is associated with resistance to infection [4]. By contrast, susceptible mice expand $CD4^+$ T lymphocytes belonging to the Th2 subset, which secretes IL-4, IL-10 and IL-13 [5]. In human and dog, resistance to visceral leishmaniasis is also associated with the generation of a Th1 response

Abbreviations: MIDGE, minimalistic, linear expression vectors; NLS, nuclear localization signal

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or a mixture of Th1 and Th2 cytokine patterns. Here, the absence of a Th1 response is associated with chronicity of the disease and high mortality in untreated cases [4]. Taken together these findings show that, as in the mouse model, the outcome of the disease appears to be determined and regulated by the balance between the two T cell populations [6,7].

Several antigens have been used in experimental vaccination trials in murine leishmaniasis, achieving various levels of protection. Among these are *L. major* gp63 [8], gp46 [9], p4, p8 [10] and LACK [11]. The LACK antigen is a 36 KD protein highly conserved among related *Leishmania* species and is expressed in both the promastigote and the amastigote forms of the parasite. LACK is a preferential target for the early anti-parasite immune response [12]. The infection induces a strong anti-LACK response and the early activated LACK reactive cells exhibit a Th2 phenotype [13]. Immune interventions aimed at modifying the T cell repertoire may be used to alter the course of an infection. The option to modulate this rapid anti-LACK immune response by exogenous IL-12 or IFN-γ suggests that these cells are not yet fully mature Th2 cells and so can be redirected [13]. Furthermore, vaccination trials with soluble leishmania antigens or a single LACK protein in the presence of recombinant IL-12 have been shown to induce a protective Th1 response [13,14]. Thus, it seems that deviation to Th1 of the strong and specific Th2 immune response against LACK in Balb/c mice induces resistance to infection [15].

During the last decade, DNA immunization has been promoted as a new alternative for achieving specific immune responses. Protective responses with DNA vaccines against several pathogens have been demonstrated [16,17]. The ability of plasmid DNA encoding specific antigens to induce both $CD4^+$ and $CD8^+$ T cell responses could be of particular use for protection against diseases that require cell mediated immunity, including leishmaniasis. The low production costs and the high structural stability of DNA make DNA vaccination a very attractive tool for immunization. On the other hand, recombinants of vaccinia virus (rVV) expressing different foreign antigens have been successfully used to elicit protective immunity to a variety of pathogens [18–21]. For instance we have shown that immunization with rVV expressing the *L. amazonensis* gp46 antigen elicits significant protection and long-term immunological memory in BALB/c mice [22]. Experiments in mice and other models have shown that a combination of these two approaches (priming with DNA and boosting with recombinant vaccinia expressing the same protein) is associated with the highest immunogenicity and protective efficacy against several infectious agents. Using this approach we have demonstrated a good protection against *L. major* in mice with the LACK antigen [23].

Expression plasmids typically used in DNA-based vaccination usually contain a transcription unit and bacterial sequences necessary for plasmid amplification and selection. In order to avoid some of the potential and principal problems associated with the use of these plasmids, we have designed minimalistic, immunogenically defined gene expression (MIDGE) vectors that contain only the eukaryotic gene expression cassette [24]. Thus, MIDGE constructs carry no sequence elements other than those needed for gene transfer. Their ends provide unique and selective target sites for the coupling of modifying molecules. To assess their utility for vaccination against leishmaniasis, MIDGE vectors expressing the LACK antigen from *L. infantum* were constructed. A nuclear localization signal (NLS) peptide derived from the T antigen of the SV40 virus was covalently linked to modification target sites at the ends of the LACK expressing MIDGE vectors. NLS peptides conjugated to plasmids have been shown to increase transfection efficiency which could result in enhanced immunogenicity [25–27]. NLS-modified and non-modified MIDGE vectors were investigated in their ability to confer protection against challenge infections, and compare in this aspect to various schemes of plasmid-based and rVV vaccinations.

Here, we show that immunization of mice with MIDGE vectors coding for p36/LACK, induced the same level of protection against challenge with *L. major* than equimolar amounts of plasmid coding for the same antigen. Most importantly, two doses of NLS-modified MIDGE conferred full protection against the same antigen, resembling the results obtained by priming with plasmid DNA and boosting with rVV, both expressing LACK.

2. Material and methods

2.1. Construction of plasmids, MIDGEs and modified MIDGEs

The cDNA encoding the LACK protein from *L. infantum* was obtained as described previously [11] and inserted downstream of the CMV promoter in the *Eco*RV site of the pcDNA3.1 expression vector (Invitrogen, San Diego, CA). The coding sequence of LACK/p36 was subcloned into the *Sst*I and *Kpn*I sites of the pMOK plasmid (Mologen, Berlin, Germany) generating the plasmid pMOK-p36. The MIDGE-p36 construct was derived from the pMOK-p36 plasmid after complete digestion with *Eco31*I (MBI Fermentas, Vilnius, Lithuania). The ends were used to ligate hairpin oligodeoxyribonucleotides (ODNs) with T4 DNA ligase. The mixture was concentrated and treated with *Eco31*I and T7 DNA polymerase in the absence of deoxyribonucleotides. The DNA was purified by anionic exchange column chromatography (Merck EMD-DMAE, sodium phosphate pH 7.0–0.1 M NaCl).

The NLS peptide (PKKKRKVEDPYC, a generous gift from Dr. P. Henklein, Charité, Berlin, Germany) was coupled to one of the hairpin ODN in two steps as previously described [24]. The resulting NLS-coupled ODN was purified. The plasmid pMOK encoding the surface antigen of the hepatitis B virus (HBsAg) was used as a control.

2.2. Construction of recombinant vaccinia viruses (rVV)

The cDNA encoding p36 from *L. infantum* was cloned into the pSC11 VV insertion plasmid under control of the p7.5 early/late viral promoter in the thymidine kinase (TK) locus. This plasmid contains the *E.coli* β-galactosidase gene under the control of the p11 viral late promoter (pSCp36). Vaccinia virus (VV) recombinants were derived from the wild type Western Reserve (WR) strain, and rVVp36 was prepared by transfecting with the insertion plasmid WR-infected BSC-40 cells [20]. The recombinant viruses were harvested 48–72 h post-infection and selected after plaque assay by addition of X-Gal to the agar. β-Galactosidase-producing plaques picked and re-plated three times and amplified following standard procedures [23,28].

2.3. Cells

African green monkey cells (BSC-40) and HeLa cells were cultured in Dulbecco's modified Eagle medium (DM-EM) supplemented with 10% newborn calf serum (NCS, Gibco BRL, Paisley, UK). Viruses were grown in HeLa and titrated in BSC-40 cells. COS-7 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS, Gibco BRL, Paisley, UK).

2.4. Mice

Female 6–8-week-old BALB/c mice, maintained under pathogen-free conditions, were obtained from the facilities at the Centro Nacional de Biotecnología (Madrid).

2.5. Parasites and reagents

L. major (WHOM/IR/-173) was a kind gift from Dr. N. Glaichenhaus (CNRS, Valbonne, France). Promastigotes were cultured at 27 °C in Schneiders medium (Gibco BRL, UK) supplemented with 20% fetal calf serum and antibiotics. Parasites were expanded in BALB/c mice and *L. major* amastigotes were obtained from a hind footpad lesion. After transformation from amastigotes to promastigotes, a synchronized culture was established for 9 days until parasites were in a late stationary phase.

2.6. LACK/ p36 expression in transiently transfected cells

3×10^5 COS-7 cells were transiently transfected with equimolar amounts of the different DNA vectors (pMOK-p36: 4 μg; MIDGE-p36 and MIDGE–NLS-p36: 2.2 μg) using Lipofectamine transfection reagent (Gibco BRL, UK). One day later expression of LACK/p36 was detected by Western blot analysis of whole cell lysates. The Western blot was reacted with an anti-p36 rabbit polyclonal antibody as previously described (23). For band quantification, the Western blot was digitized and analyzed with the NIHimage program (National Institutes of Health, Bethesda Maryland). After equalization of the image, the mean density was measured for each band using a selection of fixed area and shape.

2.7. Immunization and infectious challenge

Groups of BALB/c mice ($n = 10$–11) were primed intradermally (i.d.) in the back with 100 μg per mouse of pMOK-p36 or pMOK-HBsAg in 100 μl volume. The groups primed with MIDGE-p36 and MIDGE-p36-NLS received 54.8 μg per mouse which is an equimolar concentration of the plasmids (Table 1). Two weeks later (14 d.p.i.) mice were boosted either i.d. in the back with the same amount of DNA or intraperitoneally (i.p.) with rVVp36 (5×10^7 pfu per mouse). The non-immunized group received the same volume of buffer (150 mM sodium phosphate). Three weeks after boosting (34 d.p.i.), three mice per group were sacrificed and sera were obtained. The following day (35 d.p.i.), mice ($n = 7$–8) were challenged subcutaneously in the right hind footpad with 5×10^4 live stationary phase *L. major* promastigotes. Lesion development at the inoculation site was measured weekly with a digital caliper (Mauser Digital, Switzerland) and expressed as the increase in thickness of infected versus uninfected hind foot. Mice were sacrificed at week 8 post-challenge and serum, lymph nodes and spleen collected.

2.8. Evaluation of cytokine production

Cytokine levels in cell culture supernatants were determined by ELISA. Single cell preparations from spleens were plated in triplicate at 4×10^6 cells/ml in 24 well plates (Nunc, Denmark). Soluble antigenic peptide (2 μg/ml) (a kind gift from Dr. N. Glaichenhaus [29]), p36 protein (2 μg/ml) prepared as previously described [11], soluble leishmanial antigen (4 μg/ml) and ConA (2 μg/ml) were added in a final volume of 2 ml/well. Supernatants were harvested after 48 and 72 h and stored at −80 °C until used. IFN-γ and IL-4 levels were assessed by specific ELISA using capture and

Table 1
Immunization regimes in mice

Group	Prime[a]	Boost[a]
1	pMOK-p36 (100 μg)	pMOK-p36 (100 μg)
2	MIDGE-p36 (54.8 μg)	MIDGE-p36 (54.8 μg)
3	MIDGE-p36-NLS (54.8 μg)	MIDGE-p36-NLS (54.8 μg)
4	pMOK-HBsAg (100 μg)	pMOK-HBsAg (100 μg)
5	pMOK-p36 (100 μg)	rVVp36 (5×10^7 pfu)
6	MIDGE-p36 (54.8 μg)	rVVp36 (5×10^7 pfu)
7	MIDGE-p36-NLS (54.8 μg)	rVVp36 (5×10^7 pfu)
8	Buffer[b]	Buffer[b]

[a] Injections were intradermic (i.d.) for DNA or intraperitoneal (i.p.) for vaccinia virus. Booster injections were given 2 weeks after priming.
[b] Mice from this group received 2 i.d. doses of 150 mM sodium phosphate buffer.

secondary antibodies from Pharmingen (Becton Dickinson, USA) following the manufacturer's instructions.

2.9. Detection of total IgG antibodies against p36

One day before challenge and 7 weeks post-challenge, serum was collected from each group of animals and specific anti-p36 IgG antibodies and their isotypes (IgG1 and IgG2a) were analyzed by ELISA. In brief, 96-well Maxisorp plates (Nunc, Denmark) were coated overnight at 4 °C with recombinant p36 (5 μg/ml) prepared as previously described [11]. Serum samples were diluted 5- and 10-fold in blocking buffer (1% BSA in PBS-T), added in 50 μl/well and incubated 1 h at 37 °C. Peroxidase-conjugated goat anti-mouse total IgG, IgG1 or IgG2a (Southern Biotechnology Associated, Birmingham, AL) was added and incubated for 1 h at 37 °C. Plates were then reacted with peroxidase substrate OPD (Sigma, St Louis, MO) and absorbance was read at 492 nm on a Labsystem Multiskan Plus plate reader (Tecan Maguellan, Sunrises).

3. Results

3.1. LACK/p36 encoding MIDGE vectors

The p36 encoding sequence was inserted into pMOK generating the plasmid pMOK-p36 (Fig. 1A). In this vector the LACK/p36 protein is expressed under the control of promoter and enhancer sequences from the immediate early region of HCMV. LACK/p36 expression was improved in the pMOK vector by introducing an intron sequence between the CMV promoter and the LACK/p36-encoding sequence. pMOK-p36 contains bacterial sequences required for plasmid amplification and selection (Kan^R).

The MIDGE-p36 construct was generated from the plasmid pMOK-p36 as described in Section 2 (Fig. 1A).

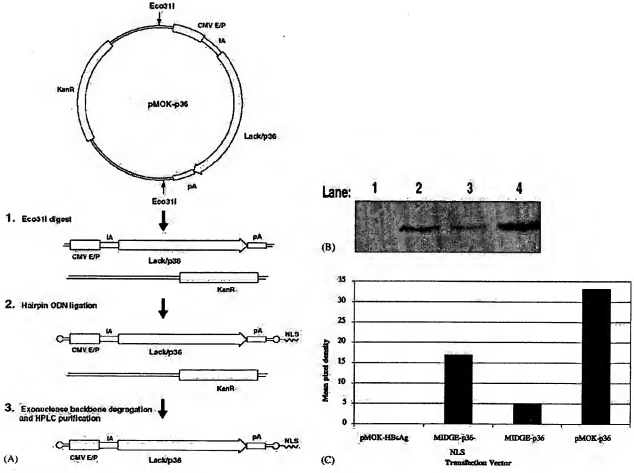

Fig. 1. Construction of MIDGE vectors and expression of LACK/p36 from different constructs. (A) The LACK-encoding pMOK plasmid was used to generate the LACK-encoding MIDGE constructs. By using NLS-modified hairpin ODN we generated the MIDGE-p36-NLS construct; (B) Expression of LACK/p36 protein in transiently transfected COS-7 cells. Cells were transiently transfected with equimolar amounts of DNA (pMOK-p36 and pMOK-HbsAg: 4 μg; MIDGE-p36 and MIDGE-p36-NLS: 2.2 μg) using lipofectamine transfection reagent. The expression of the protein was detected by Western blot analysis of lysate cells. One representative experiment is shown. Lane 1: pMOK-HBsAg; lane 2: MIDGE-p36-NLS; lane 3: MIDGE-p36; lane 4: pMOK-p36; (C) The figure shows the result of band densitometry expressed in arbitrary units (see Section 2).

MIDGE-p36 is a linear construct with covalently-closed ends. This expression unit contains only the promoter, intron, LACK/p36 encoding, and polyadenylation sequences (Fig. 1A). The linearity of the construct was confirmed by digestion with an appropriate restriction enzyme that has a single recognition site in the construct and generated two DNA fragments of the expected size (data not shown). In order to increase transfection efficiency and the expression of the antigen, we linked to the LACK expressing MIDGE vector a nuclear localization sequence signal (NLS) derived from the T antigen of the SV40 virus. The NLS peptide was coupled to one of the hairpin ODN in two steps as previously described [24].

3.2. Efficient LACK/p36 expression in cells transiently transfected with LACK/p36-encoding plasmid, MIDGE and MIDGE–NLS

Transient expression of p36 from plasmid pMOK-p36, the corresponding MIDGE construct and MIDGE-modified with the NLS sequence was tested in vitro in COS-7 cells transfected with DNA. The transfection was done using equimolar concentrations of each construct and the level of LACK/p36 expression was measured in lysed cells (Fig. 1B). The MIDGE construct expressed about six-fold less LACK/p36 protein than the plasmid (Fig. 1B and C). By contrast, NLS-modified MIDGE expressed a higher level of protein than unmodified MIDGE, but two-fold less than plasmid (Fig. 1C).

3.3. Efficacy of MIDGE vectors to confer protection against leishmaniasis

The efficacy of MIDGE vectors to trigger a protective immune response was tested in a murine model of *Leishmania* infection. For these studies, we compared protocols based on the use of a conventional plasmid and simple or NLS-modified MIDGE vectors (Table 1). Mice were primed with different constructs encoding the p36 antigen from *L. infantum*. Fourteen days later, half of the groups were boosted with rVVp36 and the others with the three different DNA vectors (Table 1). Three weeks after boosting, all animals were challenged with 5×10^4 late stationary phase *L. major* promastigotes in the right hind footpad (see Section 2). Progression and size of the lesion were measured weekly. Mice were sacrificed 8 weeks after challenge because of the large size of the lesion in control groups.

3.3.1. Priming/boosting with MIDGE vectors confers similar protection against L. major challenge than priming/boosting with a classical plasmid

All immunized groups showed several degrees of protection along the 8 weeks following the challenge (Fig. 2A). The protocol based on priming and boosting with MIDGE vector induced the same level of protection than that observed with the protocol based on priming and boosting with plasmid (Fig. 2A). Thus, both groups presented a reduction in lesion size of approximately 45% 8 weeks after challenge (Table 2).

3.3.2. Priming/boosting with MIDGE-p36-NLS confers high protection against L. major infection

When we compared the above protocols with priming/boosting with MIDGE–NLS, we observed a higher reduction in lesion size with the latter protocol (Fig. 2A). The overall protection was almost 80% compared to the control group immunized with plasmids coding for an irrelevant antigen (Mann–Whitney test, $P = 0.0056$) (Table 2). By following the progression of the disease weekly, both MIDGE and MIDGE–NLS showed a good level of

Table 2
Specific IgG2a/IgG1 ratios pre- and post-challenge, lesion size and lymph node weight at week 8 after challenge

Groups	Pre-challenge	Post-challenge		
	IgG2a/IgG1[a]	Lesion size[b] (%)	Lymph node weight[c] (mg)	IgG2a/IgG[a]
pMOK-p36/pMOK-p36	–	55	70 ± 17	0.9
MIDGE-p36/MIDGE-p36	–	56	75 ± 07	1.0
MIDGE-p36-NLS/MIDGE-p36-NLS	–	21*	54 ± 32	1.3[d]
pMOK-HBsAg/pMOK-HBsAg	–	100	74 ± 14	1.0
pMOK-p36/rVVp36	4.25	31**	45 ± 19***	1.66[d]
MIDGE-p36/rVVp36	5.76	62	93 ± 29	1.0
MIDGE-p36-NLS/rVVp36	3.98	47	71 ± 20	1.3[d]
Buffer/buffer[e]	–	85	73 ± 23	0.96
Positive control[f]	0.5			

[a] IgG2a/IgG1 represents the ratio of the mean absorbance values at 492 nm of anti-LACK specific antibodies from each immunization group determined by an indirect ELISA.
[b] Percentage of lesion size development relative to the lesion size in the group inoculated with two doses of pMOK-HBsAg at 8 weeks after challenge.
[c] Eight weeks after challenge 7–8 mice were sacrificed and each draining lymph node was extracted and weighed. Means ± S.D. are shown.
[d] Groups of mice with an IgG2a/IgG1 ratio > 1 at week 8 post-challenge are underlined.
[e] These mice received two doses of 150 mM sodium phosphate.
[f] This represents the IgG2a/IgG1 ratio in a non-immunized mouse infected with 5×10^4 live stationary phase *L. major* promastigotes.
* $P = 0.0056$.
** $P = 0.0042$.
*** $P = 0.0023$.

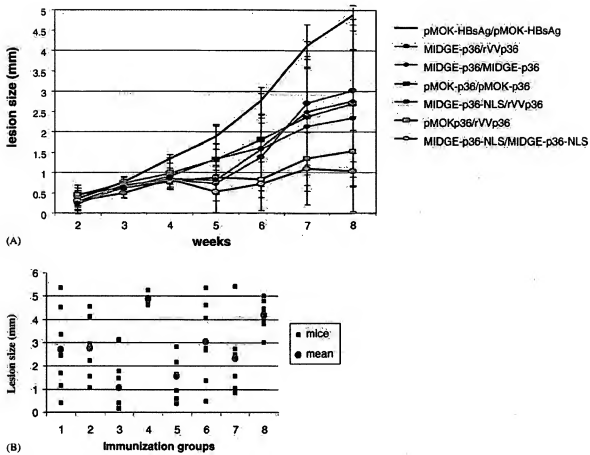

Fig. 2. Course of *L. major* infection in mice. (A) To examine the protective efficacy of the vectors, 3 weeks after boosting, groups of 7–8 mice were challenged s.c. in the right hind footpad with 5×10^4 live stationary phase *L. major* promastigotes. The state of the infection was followed weekly. Lesion size was determined by measuring the thickness of the right footpad with a digital caliper, and expressed as the difference of thickness between the infected and the uninfected collateral footpads. The figure shows the mean values ± S.D. of lesion size in each immunization group measured at weekly intervals; (B) Lesion development at 8 weeks post-challenge. Black squares represent the lesion score for each individual mouse and grey dots the mean value for each immunized group.

protection up to week 5 post-challenge (Fig. 2A). However, after this time point, the lesion developed in the group primed/boosted with MIDGE-p36 while it did not progress in the group primed/boosted with MIDGE-p36-NLS. This was manifested after examination of lesion size in individual animals (Fig. 2B). In fact, 57% (four out of seven) of the animals from this group had virtually no lesion (<0.5 mm) 8 weeks after challenge.

3.3.3. The protocol based on priming/boosting with MIDGE-p36-NLS induces at least as good protection against L. major than priming with plasmid DNA vector and boosting with rVVp36

Although vaccination with DNA alone has proven effective in animal models, it has been recently shown that a protocol based on priming with DNA vector and boosting with vaccinia virus recombinants expressing different genes induces high protection upon challenge with several pathogens including *Leishmania* [20,23,30–36]. We, therefore, compared the efficacy of priming/boosting between classical plasmid and MIDGE vectors with rVV expressing p36. As shown in Fig. 2A, the extend of protection triggered by the protocol based on priming/boosting with MIDGE-p36-NLS was similar to that induced by priming/boosting with pMOK-p36 and rVVp36, showing not statistically significant differences between both groups. At the end of the experiment, the average reduction in lesion size was almost 80% in the group immunized with two doses of MIDGE-p36-NLS (Table 2), whereas the group immunized with pMOK-p36/rVVp36 presented an average reduction of 64% compared to the group immunized with plasmids coding for an irrelevant antigen (Mann–Whitney test, $P = 0.0056$ and $P = 0.0042$, respectively) (Table 2). The difference in lesion size between MIDGE-p36-NLS/MIDGE-p36-NLS and pMOK-p36/rVVp36 immunized animals was not significant (Mann–Whitney test, $P = 0.22$).

When used only for priming, both MIDGE and MIDGE–NLS showed a good level of protection up to week 5 post-challenge (Fig. 2A). However, after this time point,

the lesion developed very rapidly in the group primed with MIDGE-p36 and more progressively in the one primed with MIDGE-p36-NLS (Fig. 2A), showing a reduction in lesion size of 38 and 53%, respectively, 8 weeks after challenge as compared to the group immunized with two doses of pMOK-HBsAg (Mann–Whitney test, $P = 0.52$) (Table 2).

3.4. Reduction in lesion size correlates with small lymph nodes

Reduction in lesion size indicates that parasite replication is diminished in the local lymph node. To test that, each popliteal lymph node from the infected leg was extracted and weighted. Average lymph node weight was significantly lower in the two most protected groups (Table 2). In the group immunized twice with MIDGE-p36-NLS the average lymph node weight was almost 30% lower than that in controls and 40% lower in the group immunized with pMOK-p36 and rVVp36 (Mann–Whitney test, $P = 0.25$ and 0.0023, respectively).

Interestingly, the group immunized with MIDGE-p36/rVVp36 had the largest lymph node of all, even more than controls. This may reflect the rapid growth of the lesion in this group over the last 3 weeks of the analysis (Fig. 2A). Alternatively, a large lymph node could reflect an active lymphocyte proliferation. This possibility could not be ruled out directly due to low number of cells recovered from the local lymph node. However, phenotypic analyses of splenocytes from all groups showed no differences in the percentage of any cell subset or activation market (data not shown). These suggested the lack of a general immune activation.

3.5. Humoral immune responses before L. major challenge

Since the outcome of the disease may be determined by the extent and the type of the immune response, we decided to check the antibody response before the challenge. Three weeks after boosting, serum was collected from each group of animals and the specific anti-p36 immunoglobulin G levels and their isotypes (IgG1 and IgG2a) were evaluated. As positive control, two sera from animals infected with *L. major* were used. Specific anti-p36 total IgG was only detected in sera from animals immunized with protocols based on priming with DNAp36 (pMOK, MIDGE or MIDGE–NLS) and boosting with rVVp36 (Fig. 3). The main isotype produced was IgG2a indicating a prevalent Th1 immune response (Table 2). By contrast, control animals developed a characteristic Th2 immune response (Table 2).

3.6. Humoral and cellular immune responses after L. major challenge

Since activation of a Th1 type of immune response may be required to induce protection against *Leishmania* infection, we were interested in characterizing the changes in immunoglobulin isotypes and cytokine production following challenge in animals. Spleens and sera were taken from sacrificed mice and specific IgG antibodies, and IFN-γ and IL-4 production were evaluated. Analysis of specific IgG levels and their isotypes revealed that all groups had developed anti-p36 total IgG as expected because all of them had been challenged with the parasite. The levels were slightly higher in the groups primed and boosted with MIDGE-p36 and MIDGE-p36-NLS (Fig. 3). Most importantly, the highest overall IgG2a/IgG1 ratios were present in the groups that showed the highest levels of protection, indicating a prevalence of a Th1 immune response (Table 2).

In order to further correlate the generation of a Th1 response with protection, pooled splenocytes from each group were stimulated with either recombinant p36 protein, an immunodominant class II-specific peptide from this protein [29], a lysate of *Leishmania* parasite or Concavalin A as positive control. IFN-γ (a Th1 cytokine) and IL-4 (Th2) production was determined in the supernatants 72 h after stimulation by ELISA. No IL-4 could be detected in any of the

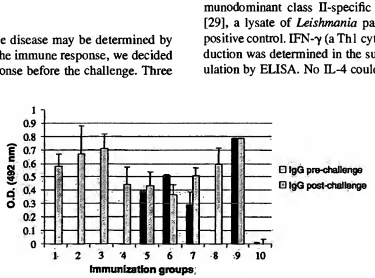

Fig. 3. Specific IgG antibodies before and after challenge with *L. major*. Sera were collected 1 day before challenge with *L. major* parasite and 8 weeks post-challenge. Specific anti-p36 IgG antibodies were analysed by ELISA. Black bars represent the mean ± S.D. of three mice per group (pre-challenge) and grey bars represent the mean ± S.D. of 7–8 mice per group (post-challenge). Groups 1–8 are defined in Table 1. Group 9 represents serum from a non-immunized animal infected with 5×10^4 live stationary phase *L. major* promastigotes (the mean ± S.D. of triplicate wells is presented). Group 10 represents serum from a naive mouse (the mean ± S.D. of triplicate wells is presented). Serum from group 9 was used to normalize both ELISAs.

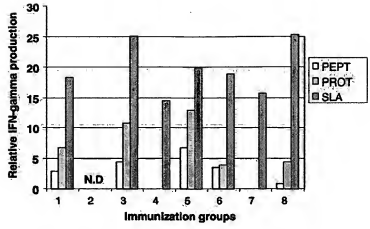

Fig. 4. IFN-γ production by in vitro stimulated splenocytes. Eight weeks after challenge, spleens were removed and pooled in each group. Cells were stimulated in vitro with recombinant LACK protein, the main immunogenic peptide from LACK [14], soluble leishmanian antigen (SLA) or ConA. Seventy-two hours post-stimulation supernatants were harvested and IFN-γ production was assessed by specific ELISA. The concentration of IFN-γ was calculated by extrapolating the absorbance value to a standard curve made with known concentrations. Values between groups were standardized by using the IFN-γ production after stimulation with ConA as the maximum value. Bars represent the percentage of IFN-γ production relative to the production after ConA stimulation. Groups 1–8 are those defined in Table 1. ND: not determined.

groups presumably due to the low sensitivity of the assay. By contrast, relatively high levels of IFN-γ were observed in all groups after stimulation with LSA. Stimulation with the p36 protein or the peptide induced a higher IFN-γ production in the groups showing the best protection (Fig. 4).

4. Discussion

DNA immunization represents a novel and interesting approach to vaccine development and immunotherapy, particularly for applications where cell-mediated immune responses are required. Several obstacles are hampering the translation of this promising technology to the clinic, including safety concerns associated to the use of conventional plasmids and the high doses required to obtain protective immunization. We and others have demonstrated that the use of plasmid DNA as priming agent followed by boosting with recombinants of vaccinia virus coding for the same antigen represents a very effective protocol for immunization and protection [20,23,30–36]. Although this strategy allows a considerable reduction in the amount of DNA required, the use of recombinant viruses abrogates some of the advantages of DNA vaccination. In the present work we have studied the ability of minimalistic, immunogenically-defined, gene expression vectors to induce protection in a mouse model of *Leishmania* infection. MIDGE vectors offer several significant advantages over conventional plasmids, such as small size, absence of antibiotic resistance genes and selective target sites for chemical linkage of peptides, proteins, sugars, etc. [24]. The efficacy of MIDGE vectors was compared to that of plasmids in several immunization protocols including boosting or not with rVV (Table 1).

The very conserved LACK antigen from *L. infantum* was cloned into both a plain MIDGE vector and a MIDGE-modified by covalently-linked nuclear localization signals (NLS) (Fig. 1A). NLS has been shown to increase the translocation of both proteins [37] and DNA [25–27] from the cytoplasm to the nucleus thus bypassing one of the main barriers for the expression of foreign DNA. To test whether this increase in nuclear transport correlated with a higher antigen expression, COS-7 cells were transfected in vitro and LACK expression was detected by Western blotting. As shown in Fig. 1B, the addition of one copy of NLS resulted in a three-fold increase in antigen expression compared to plain MIDGE. However, this expression was still lower than that achieved by transfection with an equimolar amount of plasmid coding for the same protein, indicating that increased transport to the nucleus is not sufficient to achieve high antigen expression.

MIDGE vectors expressing the LACK protein were then used for in vivo immunization of Balb/c mice and their efficacy in inducing protection was determined by challenging the mice with promastigotes from *L. major*. This is a well established model that has been extensively used to study the immunological parameters involved in *Leishmania* infection and protection as well as for the validation of vaccine candidates against this parasite [3,6]. As shown in Fig. 2, two doses of plain MIDGE-p36 confer partial but sustained protection against the challenge. This protection is identical to that induced by two doses of plasmid, but it is achieved with only half the amount of DNA (Table 1). The development of the lesion also follows the same kinetic in both groups of immunized animals (Fig. 2A) suggesting that these vectors induce both quantitatively and qualitatively similar immune responses in spite of the lower antigen expression observed in MIDGE-transfected cells in vitro

(Fig. 1B). Most importantly, the best protection against challenge was observed in the group immunized with two doses of NLS-modified MIDGE vector (Fig. 2A). In this group, four out of seven mice were completely protected at the termination of the study and only one presented a lesion of considerable size (Fig. 2B). These results were even better than those obtained by priming with plasmid and boosting with rVV, which is considered to be one of the best immunization protocols available [36,38]. In addition, both MIDGE-p36 and MIDGE-p36-NLS conferred a good but short protection against challenge with *L. major* when used as priming vectors followed by boosting with rVVp36 (Fig. 2). Mice from these groups were completely protected up to 5 weeks post-challenge but from this time the lesion developed very rapidly, particularly in the group primed with MIDGE-p36 (Fig. 2A).

Trying to understand the immunological mechanisms underlying these results, we analyzed serum from three mice in each group for specific anti-LACK IgG antibodies and their isotypes at the time of challenge. It has been previously shown by many authors that in this model of *Leishmania* infection, there is a clear correlation between resistance to infection and the development of a Th1 type of response, whereas susceptibility correlates with the development of a Th2 response [3,5]. Although protection against *Leishmania* is not mediated by antibodies [7], IgG isotypes are a valid downstream indicator of the type of T-helper immune response generated. Specific IgG antibodies were only detected in those groups receiving rVVp36 at the time of boosting and not in those receiving two doses of DNA (Fig. 3). The explanation for these results could be that LACK is a cytoplasmic protein that cannot be detected by B cells when expressed from a DNA vector. By contrast, Vaccinia virus induces lysis of infected cells [21] and so the whole protein becomes exposed to the immune system. The IgG antibodies produced by mice boosted with rVVp36 were predominantly of the IgG2a isotype (Table 2), indicating the induction of a Th1 response, as is normally the case when using rVV [21].

In contrast to these results, all mice had specific anti-LACK antibodies 8 weeks after challenge (Fig. 3), implying that replication of the parasite and cell lysis had occurred in all of them. This was also suggested by the increase in size of the local lymph node at the termination of the experiment, which was particularly important in those groups with the lowest protection (Table 2) and which could not be attributed to a higher immune response (data not shown). Interestingly, the groups immunized with two doses of any DNA vector had the highest antibody levels. This suggests that, although undetectable at the time of the analysis, DNA immunization had probably induced a low and/or transient production of antibodies that was boosted by the infection. However, as mentioned above, antibodies do not seem to be involved in protection because their level before or after challenge did not correlate with lesion development. Specific IgG isotypes were also determined at the time of sacrifice. As shown in Table 2, those groups with a higher IgG2a/IgG1 ratio (indicating a bias towards a Th1 response) were the best protected at week 8 post-challenge. To further correlate protection with the generation of a Th1 response, pooled splenocytes from every group of mice were re-stimulated in vitro with different stimuli and the IFN-γ production (a Th1 cytokine) was measured in the supernatants. Results were not very conclusive, probably because by week 8 after challenge the immune system had time to generate a response against many antigens from the parasite. As a consequence, the specific anti-LACK cellular response, important in the early stages of the infection, may be difficult to detect. This is demonstrated by the fact that splenocytes from most groups were able to produce INF-γ in response to a whole *Leishmania* lysate (Fig. 4). Nonetheless, it seems that only splenocytes from groups showing the best protection (group 3: MIDGE-p36-NLS/MIDGE-p36-NLS and group 5: pMOK-p36/rVVp36) produced a good amount of IFN-γ in response to LACK (Fig. 4). More experiments have to be done to confirm this correlation, preferably soon after the challenge because the anti-LACK response normally occurs very early after infection and so it may be at this point when generation of the right (Th1) response is critical for controlling replication of the parasite.

Altogether, we have shown that MIDGE vectors are very efficient at inducing protection against challenge in a well-studied murine model of *Leishmania* infection. Although in vitro, the plain MIDGE vector induced lower protein expression than plasmid, in vivo they both conferred similar protection. This suggests that antigen load may not be the only limiting factor when vaccinating with DNA and that MIDGE vectors have an unknown feature that makes them immunologically more efficient. Alternatively, it could be that MIDGE vectors induce higher protein expression in vivo or that the amount of antigen expressed after two doses is above the threshold required for the induction of an immune response.

When given only for priming, half the amount of both plain MIDGE and NLS-modified MIDGE vectors in combination with rVV conferred almost full protection during the first 5 weeks post-infection. After this time, however, the parasite took over and the lesion developed very quickly in these two groups. By contrast, when plasmid was used for priming, the infection was controlled over a long period of time. It is not clear why priming with plasmid DNA followed by boosting with recombinant vaccinia virus is such an effective protocol for immunization whereas both used in the opposite order are not effective. One of the most plausible explanations is that rVV given first strongly primes many anti-viral T and/or B cells and so dilute the antigen-specific ones, which are then very difficult to re-amplify by the DNA booster [21–40]. When DNA is given first, the response maybe low but specific against the cloned antigen and so can be easily amplified by the very immunogenic rVV [40]. Our results indicate that to achieve a strong and long-lasting immune response with this protocol it may be necessary to generate enough antigen during the priming step. This would

explain why plasmid was better than MIDGE vectors as priming agent and why the group primed with NLS-modified MIDGE contained the infection slightly better than the one primed with plain MIDGE. Experiments are underway to test this possibility by using larger amounts of MIDGE vectors to achieve similar antigen load than when priming with plasmid.

The most important outcome from this work was the strong protection obtained with only two doses of NLS-modified MIDGE coding for p36. To our knowledge, this is the first time that an immunization protocol based only on DNA gives comparable or even better protection than one based on plasmid DNA + rVV. This result can not be attributed to a higher antigen load induced by the MIDGE vector system because, as already emphasized, plasmid was better for protein expression at least in vitro (see above). Most likely, the presence of the NLS peptide covalently linked to the DNA is making the vector qualitatively different. In fact, when working on a different model, we have observed that immunization with NLS-modified MIDGE vectors predominantly induce a Th1 type of response in mice, even in conditions where other vectors induce a Th2 response (Moreno et al., manuscript in preparation). The basic mechanism underlying this bias is still unclear, but it could explain the good protection conferred against *Leishmania* infection, reported to be highly dependent on the generation of a Th1 response [3]. Although the design of the present experiment, more focused on protection than on basic immunological studies, does not allow for a more precise determination of the type of immune response generated, the data available suggest that the most protected groups had more Th1 response. On the basis of all the data, we can conclude that a long-lasting protection against *Leishmania* infection from immunization with the LACK antigen first requires a certain amount of antigen to trigger the specific immune response. Once generated, only if the response is of the Th1 type, the infection will be controlled.

In summary, the evidence presented here shows that MIDGE vectors are a true alternative to plasmids and recombinant viruses for protective immunization. MIDGE vectors offer great hope for the development of effective and safe vaccines against *Leishmania* and other devastating infections for which conventional vaccines have no effect.

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